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Prevalence and Sequence Diversity of a Factor Required for Actin-Based Motility in Natural Populations of *Burkholderia* Species▽†

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Actin-based motility of the melioidosis pathogen *Burkholderia pseudomallei* requires BimA. We report a high degree of conservation of *bimA* in 99 *B. pseudomallei* isolates from the area of endemicity. A geographically restricted subset of *B. pseudomallei* isolates harbored a *B. mallei*-like *bimA* allele (12.1%), confounding a differential diagnostic test based on amplification of species-specific *bimA* regions.

Burkholderia pseudomallei is a gram-negative facultative intracellular pathogen that causes melioidosis, a severe invasive disease of animals and humans that is endemic in southeast Asia and northern Australia (reviewed in reference 1). The organism can invade nonphagocytic cells, persist in phagocytes, escape endosomes, propel itself within and between cells by polar nucleation of actin, and induce cell fusion (reviewed in reference 32). *B. pseudomallei* is related to the etiological agent of glanders, and multilocus sequencing typing (MLST) (8), comparison of complete genome sequences (12, 18), and microarray analysis of gene content and transcription (15, 21) indicate that *Burkholderia mallei* is a clone of *B. pseudomallei* that has undergone gene decay. *B. pseudomallei* is a saprophyte, and environmental isolates were previously separated into two groups on the basis of their restriction and amplified-fragment profiles, ribotyping, and ability to assimilate arabinose. Those which assimilate arabinose (ara^+) were later assigned to species *Burkholderia thailandensis*, a distinction supported by MLST (8). Those which do not assimilate arabinose are associated with melioidosis and are retained in the species *B. pseudomallei*. *B. thailandensis* exhibits a substantially elevated median lethal dose relative to *B. pseudomallei* in rodents and is less adherent and invasive in cell culture models (reviewed in reference 32).

The rapid deterioration and poor prognosis of patients with melioidosis and glanders highlight a requirement for early diagnosis and intervention. PCR-based methods for discrimination of *B. pseudomallei* and *B. mallei* from ara^+ biotypes such as *B. thailandensis* have been developed based on the uneven conservation of type III secretion system genes (19, 28) and show promise in a clinical setting (16). A further PCR-based test to discriminate between *B. pseudomallei* and *B. mallei* that relies on variation in the *bimA* gene has been reported (30). BimA is required for actin-based motility of *B. pseudomallei* (27), and there are orthologues in *B. mallei* and *B. thailandensis* that can restore intracellular motility to a *B. pseudomallei bimA* mutant, despite divergence of their N-terminal sequences (26). BimA is not required for virulence of *B. mallei* in a Syrian hamster model of acute glanders (23); however, attenuation of a *B. pseudomallei bimA* mutant has been detected (M. Stevens and G. Bancroft, unpublished observations), consistent with the role of actin-based motility in the pathogenesis of *Listeria* and *Shigella* sp. infections (reviewed in reference 25).

Natural diversity exists in the prevalence and sequences of bacterial factors required for actin-dependent movement, including *Listeria* ActA (10, 17), *Shigella* and enteroinvasive *Escherichia coli* IcsA (4), *Rickettsia* sp. RickA (9, 14), and the Tir and TccP proteins of attaching and effacing *E. coli* (7, 20, 22). Such factors are not ubiquitous in all species of these genera, and the impact of polymorphisms on actin assembly, cell-to-cell spread, and pathogenesis is ill defined. The BimA orthologues described to date contain variable numbers and types of a proline-rich motif (26), and variation in the number of such motifs has been related to the efficiency of actin assembly by enterohemorrhagic *E. coli* TccP (6) and *Listeria* ActA (24). Further, the BimA protein from *B. thailandensis* was uniquely able to interact with Arp-3 (actin-related protein 3), consistent with the presence of a central and acidic domain that is absent in the BimA proteins from *B. pseudomallei* and *B. mallei* (26). Here we sampled the prevalence and sequence of BimA in clinical and environmental isolates of *B. pseudomallei*, both to evaluate the reliability of differential PCR tests based on *bimA* and to examine the conservation of motifs and

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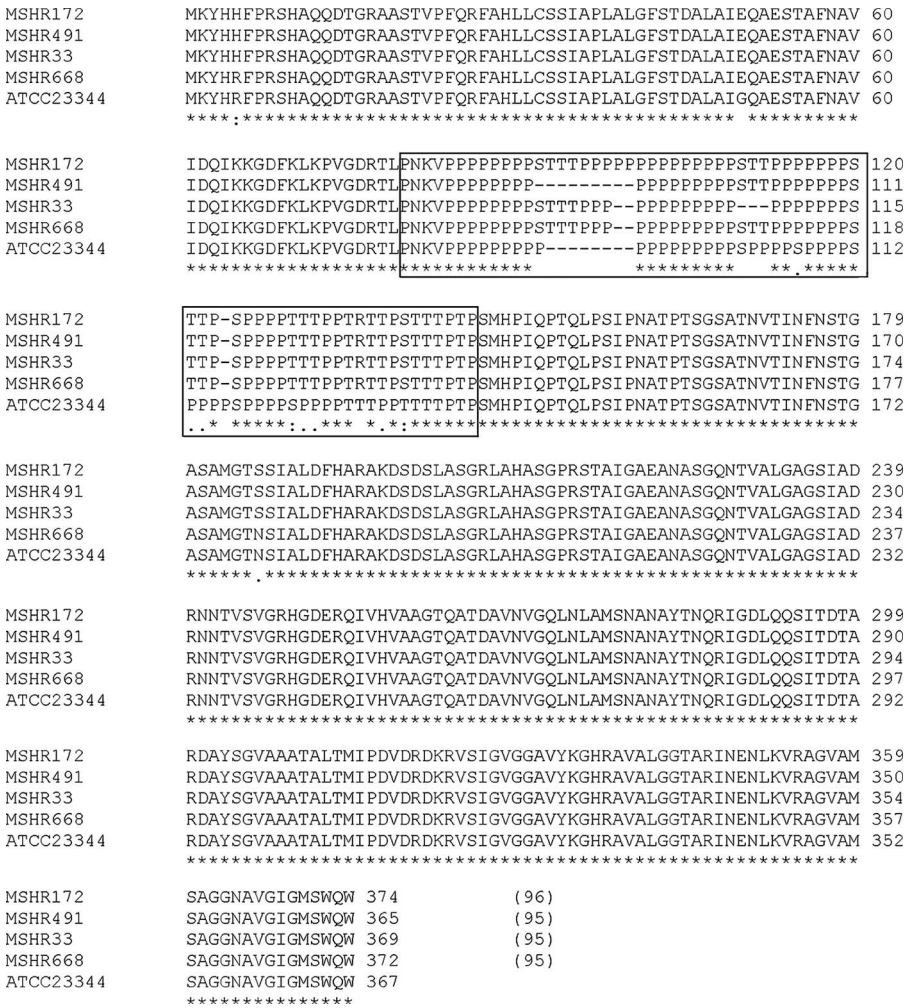

FIG. 1. Alignment of *B. mallei*-like BimA sequences derived from three Australian *B. pseudomallei* isolates with BimA sequences of *B. mallei* ATCC 23344 and *B. pseudomallei* MSHR668. Amino acid sequences were aligned using ClustalW (3). Alignment scores representing the level of identity to the *B. mallei* ATCC 23344 BimA sequence are shown in parentheses. The proline-rich region is boxed.

domains predicted to be important for the subversion of actin dynamics by this factor.

To gain insight into the extent of variation of BimA and design a strategy for amplification of *bimA* genes, we first examined *bimA* sequences in the complete or partial genomes of 18 *B. pseudomallei*, 10 *B. mallei*, and 2 *B. thailandensis* strains available at the time of writing. In all cases, a single *bimA* open reading frame was predicted to give rise to a protein of approximately the expected size for the species and no pseudogenes were detected. ClustalW alignment (3) of predicted protein sequences revealed that the BimA orthologues previously described in *B. pseudomallei* ($BimA_{Bp}$), *B. mallei* ($BimA_{Bm}$), and *B. thailandensis* ($BimA_{Bt}$) (26) are highly conserved in other representatives of the same species. However, an Australian isolate of *B. pseudomallei* (MSHR668) possessed a BimA sequence exhibiting only 54% identity to the BimA of the prototypic *B. pseudomallei* strain K96243. The BimA of strain MSHR668 is 95% identical to the BimA of the prototypic *B. mallei* strain ATCC 23344 and exhibits the same predicted domain organization. Of the BimA domains described previously (27), the membrane anchor and predicted actin monomer-binding Wiskott-Aldrich syndrome protein homology 2 (WH2) domains of the BimA from strains ATCC 23344 and MSHR668 are identical, as determined using MotifScan (5). However, differences existed in the predicted proline-rich domain, which in *B. mallei* ATCC 23344 BimA contains six PRM1 motifs (VP_{18} and five SP_4 motifs) but in the BimA of strain MSHR668 contains only two PRM1 motifs (VP_8 and SP_4) (Fig. 1). PRM1 motifs interact with profilin, which in turn recruits actin monomers to sites of assembly (reviewed in reference 13); however, the impact of the polymorphisms on BimA function remains to be elucidated.

Of the 17 predicted *B. pseudomallei* BimA sequences that resemble the prototypic $BimA_{Bp}$ from K96243, the predicted proline-rich motifs and tandem WH2 domains were conserved. The BimA of *B. pseudomallei* strain 14 contains only a single NIPVPPPMPGGGA motif, which is directly repeated in tandem in all other strains. The number of PDAST repeats (predicted phosphorylation sites for host cell casein kinase II) was also found to range from two (*B. pseudomallei* BCC215) to seven (*B. pseudomallei* MSHR305) (see Fig. S1 in the supplemental material). The central and acidic domain predicted in

TABLE 1. *B. pseudomallei* strains used in this study, their origin, MLST type, and YLF/BTFC type (where known)[a]

Strain	Origin		ST	YLF/BTFC type	Strain	Origin		ST	YLF/BTFC type
	Country	Source				Country	Source		
MSHR295	Australia	Soil			1542A	Thailand	Human		
MSHR296	Australia	Soil			1554A	Thailand	Human		
MSHR503	Australia	Soil			1842A	Thailand	Human		
MSHR491	**Australia**	**Water**	**126**	**YLF**	1943A	Thailand	Human		
MSHR145	Australia	Animal			2035A	Thailand	Human		
MSHR151	Australia	Animal			2167A	Thailand	Human		
MSHR172	**Australia**	**Animal**	**284**	**BTFC**	2506A	Thailand	Human		
MSHR408	Australia	Animal			2652A	Thailand	Human		
MSHR480	Australia	Animal	132		2659A	Thailand	Human		
MSHR571	Australia	Animal	109		2661A	Thailand	Human		
MSHR1306	Australia	Animal			2667A	Thailand	Human		
MSHR33	**Australia**	**Human**	**647**		2670A	Thailand	Human		
MSHR42	**Australia**	**Human**	**648**		2679A	Thailand	Human		
MSHR44	**Australia**	**Human**			2717A	Thailand	Human		
MSHR287	**Australia**	**Human**	**125**	**YLF**	2719A	Thailand	Human		
MSHR305	Australia	Human	36		3060A	Thailand	Human		
MSHR373	**Australia**	**Human**	**430**	**BTFC**	3065a	Thailand	Human		
MSHR387	Australia	Human			3066s	Thailand	Human		
MSHR423	Australia	Human			3069A	Thailand	Human		
MSHR432	**Australia**	**Human**		**BTFC**	3073A	Thailand	Human		
MSHR439	Australia	Human			3082B(a)	Thailand	Human		
MSHR449	**Australia**	**Human**	**126**	**YLF**	K96243	Thailand	Human		
MSHR456	**Australia**	**Human**	**113**	**BTFC**	U1065	Thailand	Human		
MSHR463	Australia	Human			U1106	Thailand	Human		
MSHR465	Australia	Human			U1710	Thailand	Human		
MSHR468	Australia	Human			U3161	Thailand	Human		
MSHR519	Australia	Human			U3232	Thailand	Human		
MSHR520	Australia	Human	36		10276		Human		
MSHR543	**Australia**	**Human**	**294**	**YLF**	L2	Laos	Soil		
MSHR640	Australia	Human	109		L4	Laos	Soil		
MSHR644	Australia	Human			L5	Laos	Soil		
MSHR659	Australia	Human			L8	Laos	Soil		
MSHR668	**Australia**	**Human**	**129**	**BTFC**	L9	Laos	Soil		
MSHR713	Australia	Human			L10	Laos	Soil		
MSHR730	Australia	Human			L11	Laos	Soil		
E8	Thailand	Soil			CS7	Cambodia	Soil		
E183	Thailand	Soil			CS8	Cambodia	Soil		
E186	Thailand	Soil			CS12	Cambodia	Soil		
E235	Thailand	Soil			CS13	Cambodia	Soil		
E237	Thailand	Soil			CS14	Cambodia	Soil		
E238	Thailand	Soil			CS15	Cambodia	Soil		
E241	Thailand	Soil			CS17	Cambodia	Soil		
E279	Thailand	Soil			1K	Vietnam	Soil		
E372	Thailand	Soil			2O	Vietnam	Soil		
E378	Thailand	Soil			3E	Vietnam	Soil		
E407	Thailand	Soil			5Q	Vietnam	Soil		
E408	Thailand	Soil			6D	Vietnam	Soil		
E411	Thailand	Soil			7E	Vietnam	Soil		
E412	Thailand	Soil			HK4	Hong Kong	Animal		
E413	Thailand	Soil							

[a] Data for strains which produced *B. mallei*-like *bimA* amplicons of 1.2 kb in the PCR screen are in boldface. MLST types of Australian isolates were obtained from http://bpseudomallei.mlst.net (8) or derived herein. The YLF/BTFC type was defined by PCR using locus-specific primers as described previously (29). ST, sequence type.

B. thailandensis E264 was found to be conserved in the only other $BimA_{Bt}$ sequence available but was absent in all other $BimA_{Bp}$ and $BimA_{Bm}$ sequences analyzed.

To determine if the prevalence and diversity of BimA encoded by sequenced genomes reflect those of BimA encoded by genomes of natural populations and to determine if the *B. mallei*-like BimA exists in other *B. pseudomallei* strains, *bimA* genes were amplified from a collection of 99 *B. pseudomallei* strains of clinical or environmental origin from the area of endemicity (Table 1). Genomic DNA was prepared by cetyltrimethylammonium bromide extraction and subjected to PCR with GoTaq Green Master Mix polymerase (Promega, Madison, WI) using the primers 5′-CTCGAATTCCATGCGTGC AATAGCTG-3′ and 5′-CTTCTCGAGTGCTTACCATTGC CAGCTCAT-3′, which amplify a 1.78-kb product representing the region 232 nucleotides upstream of the predicted start codon to 4 nucleotides downstream of the predicted stop codon of *B. pseudomallei* K96243 *bimA*. PCRs involved initial denaturation at 97°C for 3 min and 30 cycles at 97°C for 30 s and 68°C for 3 min, followed by a final extension for 10 min at

68°C. Amplicons were resolved by agarose gel electrophoresis and were obtained from all strains. Eighty-seven strains yielded an amplicon typical of the size of *bimA* from *B. pseudomallei* K96243 (87.9%), whereas 12 yielded a ca. 1.2-kb amplicon comparable in size to *bimA* from *B. mallei* ATCC 23344 (12.1%) (Table 1). Consistent with the findings of Ulrich et al. (30), the *bimA*-specific primers failed to amplify a specific product from the genomes of 53 strains representing nine genomovars of the *Burkholderia cepacia* complex (D. Kenna and J. Govan, unpublished data). Analysis of the sequenced genomes of *B. cepacia* complex strains also failed to identify *bimA* orthologues.

The *bimA* genes of three *B. pseudomallei* isolates that yielded 1.2-kb amplicons (strains MSHR33, MSHR172, and MSHR491) were amplified, restricted with EcoRI and XhoI, cloned under the control of a Ptac promoter into similarly restricted pME6032 (11), and sequenced by the Bio-Technology Service Unit, Thailand. The predicted BimA proteins of strains MSHR33, MSHR172, and MSHR491 vary slightly in length (369, 374, and 365 amino acids, respectively) and exhibit 54 to 55% identity to *B. pseudomallei* K96243 BimA yet 95 to 96% identity to the BimA of *B. mallei* ATCC 23344, indicating that they are *B. mallei*-like (Fig. 1). The $BimA_{Bm}$-like BimA proteins from strains MSHR33, MSHR172, and MSHR491 differ from those of *B. mallei* ATCC 23344 and *B. pseudomallei* MSHR668 only in the proline-rich domain, where they show greater similarity to the region in *B. pseudomallei* MSHR668 BimA than the corresponding region in *B. mallei* ATCC 23344 (Fig. 1). The $bimA_{Bp}$ and $bimA_{Bm}$ alleles may reflect distinct horizontal gene transfer events, as recently described for *Yersinia*-like fimbriae (YLF) and *B. thailandensis*-like flagellum and chemotaxis (BTFC) clusters that define distinct and geographically restricted isolates of *B. pseudomallei* (29).

Remarkably, all 12 *B. pseudomallei* strains harboring $bimA_{Bm}$-like genes were isolated from the Australian Northern Territory, consistent with the existence of a $bimA_{Bm}$-like allele in the sequenced genome of the Australian *B. pseudomallei* isolate MSHR668. A further 23 *B. pseudomallei* strains originating from Australia (65.7%) yielded a 1.8-kb $bimA_{Bp}$-like amplicon (Table 1). Previously, MLST indicated that Australian and Thai isolates of *B. pseudomallei* are distinct (2, 31). The MLST types of some of the strains tested are known (http://bpseudomallei.mlst.net) (8) (Table 1), and the sequence types of a further three *B. pseudomallei* isolates harboring the $bimA_{Bm}$ allele were determined herein. This was accomplished by sequencing internal fragments of the housekeeping genes *ace*, *gltB*, *gmhD*, *lepA*, *lipA*, *narK*, and *ndh* from strains MSHR33, MSHR42, and MSHR172 on both strands as described previously (8, 31). A phylogenetic analysis was performed to determine the relatedness of $bimA_{Bm}$ allele strains to each other and to the population of isolates from northern Australia. A neighbor-joining tree was constructed based on the concatenated sequences of the seven MLST genes for the *B. pseudomallei* strains with known sequence types in this study, together with all *B. pseudomallei* strains in the MLST database (http://www.mlst.net/) originating from northern Australia that were deposited by one of the coauthors (B. Currie). The tree generated from data for 606 isolates corresponding to 227 sequence types demonstrated that the $bimA_{Bm}$ allele strains were distributed throughout the *B. pseudomallei* population, with no obvious association with specific lineages. (see Fig. S2 in the supplemental material). All of the Australian isolates had sequence types distinct from that of *B. mallei* (ST40) (31), and there are no known reports of isolation of *B. mallei* in Australia. The *bimA* locus (BPSS1492) is not genetically linked with the YLF or BTFC clusters described by Tuanyok et al. (29), and the $bimA_{Bm}$ allele was found in strains of either the YLF or BTFC group by PCR using locus-specific primers as described previously (29) (Table 1). It will be interesting to determine whether strains of a given MLST sequence type can contain either a $bimA_{Bp}$ or $bimA_{Bm}$ allele. At this stage, it is not possible to state whether $bimA_{Bm}$ was derived by gene decay in *B. pseudomallei* or if *B. mallei* arose from a *B. pseudomallei* strain in which the $bimA_{Bm}$ allele was present, although the latter seems more plausible as MLST has demonstrated clear separation between Thai and Australian strains of *B. pseudomallei* (31). It has been suggested previously that *B. pseudomallei* may have originated in Australia and was carried by animals to southeast Asia via a land bridge that existed around 15 million years ago between Asia and the Australian-New Guinea continent (2). Further studies are under way to ascertain if the $bimA_{Bm}$-like allele is associated with severity of disease or specific clinical presentations and whether this allele is present in *B. pseudomallei* from regions of endemicity other than the Northern Territory of Australia.

Ulrich et al. (30) previously reported the development of primers (AT4 and AT5) based on the sequence heterogeneity in the 5′ end of *bimA* to generate a 250-bp *B. mallei*-specific amplicon spanning the translation initiation site. Although Ulrich et al. previously reported no amplification from *B. pseudomallei* isolates from Australia, all 12 *B. pseudomallei* isolates harboring the $bimA_{Bm}$-like allele from this study yielded a 250-bp amplicon with these primers (data not shown). There is robust evidence from API 20NE, arabinose assimilation, and latex agglutination tests that these 12 strains are indeed *B. pseudomallei*, indicating that the AT4 and AT5 primers are not suitable for distinguishing *B. pseudomallei* from *B. mallei*, at least for strains from this geographical area.

The data presented herein indicate that all *B. pseudomallei* isolates examined contain an intact *bimA* gene and reveal that an allele detected in the genome of *B. mallei* is not widespread (~12%) in *B. pseudomallei*. This allele was present in isolates from Northern Territory, Australia, but not Thailand and was not restricted to a specific sequence type. The data have important implications for the design and reliability of *bimA*-based tests to differentiate between *Burkholderia* species and highlight the existence of polymorphisms that have the potential to influence actin binding and assembly by a key factor required for intercellular spread and virulence.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been registered in GenBank accession numbers EU382734, EU437409, and EU437410.

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